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Distinct requirement for an intact dimer interface in wildtype, V600E and kinase-dead BRAF signaling

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 16 December 2011

Thank you for the submission of your manuscript to The EMBO Journal. We have now received the reports from the three referees that were asked to evaluate your study, which I copy below. As all three referees think that your manuscript is interesting and their comments are quite positive, I would like to invite you to revise it according to the referees' comments.

Without going into details that you will find below, referees #1 and #3 are very positive about your manuscript and suggest only relatively minor changes. Referee #2 remarks some more serious concerns but I would like to remind you that point #1 will not be taken into consideration, as competing manuscripts published during the review process do not negatively impact on our novelty assessment.

Along these lines, I would also like to urge to re-submit your revised version as soon as possible. Given that an overlapping report has already been published, it is in your best interest and ours to expedite the publication of your study. In this regard, if you anticipate any problem in addressing any of the referees' concerns in a timely manner, please do not hesitate to contact me.

Please be aware that acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For

more details on our Transparent Editorial Process iniciative, please visit our website:
http://www.nature.com/emboj/about/process.html

Once more, do not hesitate to contact me by e-mail or on the phone in case you have any questions.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

In this manuscript Roring and colleagues analyse the dimerisation of RAF proteins, a process important for RAF activation and that lately has been shown to have a key role in the paradoxical activation of the MAPK pathway in response to RAF inhibition.

This study thoroughly investigates the role of the dimer interface (DIF) motif, a region within the kinase domain important for dimerisation, and its requirement for efficient wild-type and mutant BRAF signalling to downstream effectors. The authors demonstrate that WTBRAF and BRAF mutants are differentially affected by DIF mutations. The authors show that whereas WTBRAF requires an intact DIF motif for activation of MEK/ERK, DIF alterations do not impair V600EBRAF kinase activity or its ability to signal to MEK/ERK. The R509HBRAF DIF mutation does not affect V600EBRAF transforming capacity as well as its anchorage independent growth. Moreover, the authors demonstrate that V600EBRAF is involved in the formation of larger protein complexes, with increased stability, when compared to WTBRAF. The authors also show that an intact DIF is required for BRAF homodimer formation but is dispensable for the formation of BRAF:CRAF heterodimers, indicating that their formation has distinct structural requirements.

The importance of RAF dimerisation and its new discovered role in paradoxical pathway activation, make this study timely and highlight that the development of efficient strategies that prevent dimer formation might constitute a feasible therapeutic strategy for RAF driven tumours. Roring et al present novel findings and provide insight into the importance of the DIF motif for signalling. The data nicely complements other studies investigating BRAF biochemistry and is recommended for publication based on the following comments.

Major comments:

1. The authors show that the introduction of R509H hardly affects MEK/ERK signalling in some gain of function mutants like V600EBRAF or insTBRAF, however it impairs ERK signalling on other activating mutants like S365A, EVDK, G469A or Q257R. Can the authors elaborate on this difference?

2. The authors should restructure Figure 1, as the order by which the mutants are shown does not correspond to the order they are referred to in the text. The authors should present first the results corresponding to the kinase domain BRAF mutations and then present their results for mutations outside the kinase domain (Q257R, S365A).

3. It would be interesting to verify if DIF mutants affect CRAF homodimer formation.

4. This study shows that mutations in the DIF motif are efficient at preventing the formation of BRAF homodimers however they do not affect the formation of RAF heterodimers which are known to be important drivers of RAF paradoxical activation upon treatment with RAF inhibitors. This fact might undermine the use of DIF mutations as a strategy to prevent RAF paradoxical activation. Can the authors comment on this point?

Minor comments:

1. In Figure 1 the authors should present the quantification of MEK phosphorylation for all the mutants studied and not only just for a few selected ones.

2. In Figure 1C and 1E the mutant R509H should be included as a control.

3. The mutants EVDK, EVDK/R509H, V600E, V600E/R509H are repeated in Figure 1C and 1E.

4. Figure 6C should include an immunoblot for RAS to show that its expression is being efficiently induced by 4-hydroxy-tamoxifen.

5. In Figure 6C the authors present two immunoblots for BRAF by using one for the HA-tagged BRAF and another one for the endogenous protein (F7) but one of them should be removed as it does not add to the study.

6. In Figure 7B the levels of immunoprecipiated BRAF protein should be similar for all the analysed samples.

7. The first sentence of the Results section lacks reference.

8. Some of the supplementary references are already included in the main text.

Referee #2

Raf proteins are part of the Ras/Erk pathway. Their Ras-dependent activation entails the dimerization of their kinase domain mediated by an evolutionarily conserved interface called the "side-to-side" interface.

In this paper, Roring et al. investigate the importance of the B-Raf dimerization interface to support the activity of various gain-of-function mutations that have been identified over the years. The central take-home message of this study is that the most frequent oncogenic mutation (V600E) found in human tumors is resilient to mutations impeding dimerization whereas WT B-Raf as well as other classes of gain-of-function mutants heavily depend on it. This information is potentially important as it could have an impact on the development on a new generation of B-Raf inhibitors to tackle B-RafV600E-dependent cancers. Unfortunately, this work suffers from a number of important shortcomings that significantly reduces its significance and scope.

Major points:

1- Although the mechanism remains unknown, the apparent resilience of B-RafV600E to interface disruption has now been reported by another group (Poulikakos et al. 2011 Nature, Nov 23; Epub ahead of print).

2- The claim that the highly oncogenic B-Raf V600E mutation does not require dimerization for activity is purely based on overexpression experiments (including the transformation assays), which may have confounding consequences.

3- Although the authors claim that the G469A mutation behaves as the V600E, which would be consistent with their model, the data shown in Fig. 1 are far from convincing as the R509H mutation seems to have a significant effect on it.

4- Even if commonly used, immunoprecipitations and BN-PAGE assays remain artificial approaches to monitor protein-protein interactions. There is no guarantee that the results obtained by these methods faithfully recapitulate the state of an interaction within a living cell. Given the higher ability of the V600E mutant to form dimers, it may well be that the apparent resilience of the V600E mutant to the disruption of the dimer interface is due to the fact that dimerization still proceeds to a low but significant level to support substantial MEK/ERK activation.

5- The entire study stands out as phenomenological and as such does not provide much novel information. While mechanistic speculation as to how a V600E could escape the need for dimer formation is provided, no attempt to directly address this is made.

6- Even though the authors conducted one experiment to show that the system is not saturated when using the V600E mutant (Fig. S1). They have to repeat those experiments with a more extended dose-response curve using lower plasmid quantities (they should decrease the levels of the V600E construct to a point where they start seeing a significant reduction in pMAPK levels). Multiple determinations and quantification (including error bars) need to be provided. In fact, it appears that those mutants that are the least affected by the interface mutations are the ones that have the strongest activity. For this reason it becomes very important to conduct titration experiments to ensure that the system is not saturated. It remains though that they would then need to show that those active mutants are truly monomeric in vivo. Otherwise, it would remain impossible to conclude anything.

Minor points;

7- At places, the authors simplify the literature. For example, on page 4; 2nd para.; line 7. They say that phosphorylation of the TVKS motif of the B-Raf activation loop leads to its restructure and cite Wan et al. 2004. There is actually no single study that demonstrated this. The Wan et al paper simply conjectured that this might happen, but they never showed it. The same statement is made on top of page 7. As a matter of fact, the phosphorylation of the indicated residues has been documented by a single group and never been reproduced (which make some researchers in this field uncomfortable with this information).

8- The authors make multiple claims of being the first to demonstrate specific points:
p. 9; top line
p. 10; top line
p, 10; 2nd parag. Line 5
p. 15; 1st parag., line 7
p. 19; third line from the bottom
These are unnecessary and a bit annoying (an original paper provides by definition novel information).

Referee #3

In this paper the authors carefully dissect the role of B-Raf homo- and heterodimerization to the activation of the ERK pathway and oncogenic transformation. The paper is well organized, the experiments are generally convincing, and the conclusions are interesting for the field. The results demonstrate that the role of Raf dimerization is much more complicated than previously thought. I just have a few comments that may serve to improve the paper.

Major points
Fig. 1A. A recent publication by Baljuls et al. (J Biol Chem. 2011 May 6;286(18):16491-503) shows that the three amino acids immediately N-terminal of the DIF are also involved in Raf dimerization. The authors may consider extending their definition of the DIF motif to include these amino acids.

Fig. 4. In the previous publications about the paradoxical activation of the ERK pathway by Raf inhibitors the effects of PLX4032 tended to behave different than those of sorafenib. Thus, a comparison of PLC4032 and sorafenib in regard to the DIF mutants would be very interesting.

Fig. 7B. These results suggest that the binding sites for homo- and heterodimerization are different. This could allow the formation of ternary or even higher order complexes including homo- and heterodimers. Is that the case?

It would be interesting to compare the effects of some of the DIF mutants in cell lines, such as melanoma, where the Raf inhibitors have been previously studied.

Minor points
p.3. "The RBD mediates the interaction with Ras-GTP through a conserved arginine residue (R188 in B-Raf)." This sentence should be changed as in the current form it suggests that R188 is the sole mediator of the RBD-Ras interaction, which is not correct.

p.3. "Displacement of 14-3-3 from the CR2 and subsequent dephosphorylation of S365 (or its equivalent) is a key step in Raf activation (Rodriguez-Viciana et al., 2006a)." This already has been shown previously by Abraham et al., J Biol Chem. 2000 Jul 21;275(29):22300-4; Jaumot & Hancock, Oncogene. 2001 Jul 5;20(30):3949-58; Dhillon et al., EMBO J. 2002 Jan 15;21(1-2):64-71.

1st Revision - authors' response 01 March 2012

Referee #1

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This study thoroughly investigates the role of the dimer interface (DIF) motif, a region within the kinase domain important for dimerisation, and its requirement for efficient wild-type and mutant BRAF signalling to downstream effectors. The authors demonstrate that WTBRAF and BRAF mutants are differentially affected by DIF mutations. The authors show that whereas WTBRAF requires an intact DIF motif for activation of MEK/ERK, DIF alterations do not impair V600EBRAF kinase activity or its ability to signal to MEK/ERK. The R509HBRAF DIF mutation does not affect V600EBRAF transforming capacity as well as its anchorage independent growth. Moreover, the authors demonstrate that V600EBRAF is involved in the formation of larger protein complexes, with increased stability, when compared to WTBRAF. The authors also show that an intact DIF is required for BRAF homodimer formation but is dispensable for the formation of BRAF:CRAF heterodimers, indicating that their formation has distinct structural requirements.

The importance of RAF dimerisation and its new discovered role in paradoxical pathway activation, make this study timely and highlight that the development of efficient strategies that prevent dimer formation might constitute a feasible therapeutic strategy for RAF driven tumours. Roring et al present novel findings and provide insight into the importance of the DIF motif for signalling. The data nicely complements other studies investigating BRAF biochemistry and is recommended for publication based on the following comments.
We would like to thank referee #1 for his/her very positive and helpful criticism. Our point-by-point response to the comments is provided below.

Major comments:

1. The authors show that the introduction of R509H hardly affects MEK/ERK signalling in some gain of function mutants like V600EBRAF or insTBRAF, however it impairs ERK signalling on other activating mutants like S365A, EVDK, G469A or Q257R. Can the authors elaborate on this difference?
We would like to thank the reviewer for this interesting question that arose from the panel of various gain-of-function mutants in our study. As we discuss on p. 20/21 of our manuscript (old sentences and the new blue ones), we think that the most likely explanation for this phenomenon is being sought in the impact these mutations have on the overall conformation of the B-Raf kinase domain as discussed in previous publications (Eisenhardt et al., 2011; Wan et al., 2004). In essence, we suggest that the kinase domains of the naturally occurring high-activity oncoproteins (B-Raf^{V600E}, B-Raf^{insT} and B-Raf^{G469A}, Figure 1H) with mutations in either the P- or activation loop are locked in their active conformation in a more stable manner compared to the artificial (EVKD) and/or non-P- or non-activation loop mutants (Q257R, S365A, E586K, CAAX). Based on this assumption, positive regulatory requirements in the activation cycle, including the postulated allosteric activation mechanism that is associated with dimerisation (see our discussion on p. 20/21 for details) are more likely to become superfluous. In contrast, gain-of-function-mutations residing outside of the P- and

activation loops such as S365A and Q257R relieve the auto-inhibition imposed by the N-terminal moiety and therefore prime B-Raf for activation only. Consequently, these mutants would still rely on the DIF-mediated allosteric activation mechanism to transfer the kinase domain in a fully active state. A similar explanation could be applied to the B-Raf^{CAAX} mutant. Furthermore, B-Raf^{E586K} is not as independent as B-Raf^{V600E} or B-Raf^{G469A} from regulatory requirements as the former still requires the presence of the phosphorylatable S729 residue in the core of the C-terminal 14-3-3 binding motif (our unpublished data). In contrast to B-Raf^{V600E}, B-Raf^{E586K} is also still dependent on N-region charge (Emuss et al., 2005). Although the EVKD mutation is also likely to disrupt the hydrophobic interaction between F467 and V600, we posit that this artificial double-mutation confers a less stable active conformation than V600E. This assumption is supported by our observation that B-Raf^{EVKD} displays a lesser *in vitro* kinase activity than B-Raf^{V600E} (our unpublished data). Similarly, Wan *et al*., (2004) show that G469A is less active than V600E, which might explain the higher impact of the R509H dimer interface mutation on B-Raf^{G469A}. We are well aware that our explanations rely only on the structural comparison of the wildtype and V600E kinase domains (Wan et al., 2004) and indirect functional assays as the structure of all other mutated B-Raf kinase domains have not been published yet. However, we feel that also the discovery of distinct signalling qualities of B-Raf^{V600E} and B-Raf^{EVKD}, which was believed in the field to mimic B-Raf^{V600E}, is a strong point of our study and will stimulate further research in this direction, e.g. in terms of availability of new crystal structures such as B-Raf^{G469A}.

2. The authors should restructure Figure 1, as the order by which the mutants are shown does not correspond to the order they are referred to in the text. The authors should present first the results corresponding to the kinase domain BRAF mutations and then present their results for mutations outside the kinase domain (Q257R, S365A).

We would like to thank the reviewer for this suggestion and we did our best to restructure Figure 1 and the accompanying result section. In the new Figure 1 and the accompanying Supplementary Figure S2, the results corresponding to the kinase domain BRAF mutations (EVKD, V600E, G469A, E586K) are now followed by the results for mutations outside the kinase domain (Q257R, S365A, CAAX motif). However, a complete restructuring, which would also place the insT mutant before the non-CR3 mutants would be impossible without gel sclicing or time-consuming re-running of gels.

3. It would be interesting to verify if DIF mutants affect CRAF homodimer formation.

We would like to thank the reviewer for this excellent suggestion. Therefore, we generated Raf-1^{R401H} and, as Poulikakos *et al*., (2010) reported that the isolated kinase domain of Raf-1^{R401H} is unstable, a Raf-1^{R401A} expression construct as well. In a preliminary experiment, we confirmed recent observations by Baljuls *et al*., (2011) that the Raf-1^{R401A} mutant is not reduced in its homo-dimerisation potential (Raf-1^{R401H} was not analysed in their study). However, as we found that the full-length Raf-1^{R401H} mutant displayed a very similar stability as the wildtype Raf-1 protein and as all experiments in the B-Raf context were made with the histidine substitution, we focused on this mutant. As shown below as well as in the new Figure 6 and as described on p. 16, we observed the following:

i.) Introduction of the R401H mutation into one protomer does not affect the homo-dimerisation of Raf-1 as we observed for Ras-induced hetero-dimers between B-Raf$^{D594A/R509H}$ and endogenous Raf-1^{wt} (Figure 6D).

ii.) While MEK phosphorylation was strongly enhanced by ectopically expressed wildtype Raf-1 in combination with oncogenic K-Ras^{G12V}, expression of Raf-1^{R401H} quenched the MEK phosphorylation elicited by K-Ras^{G12V} below that of cells expressing this GTPase mutant alone (Figure 6F and below). This dominant-negative effect is in full agreement with the behavior of B-Raf^{R509H} in MEFs either stimulated with EGF (Figure 2A) or containing uncaged ERTmRasV12 (Figure 6C). We will refer to this finding in the discussion (p. 23) as well as we regard it as another evidence for a role of the DIF in the allosteric activation of mammalian Raf isoforms.

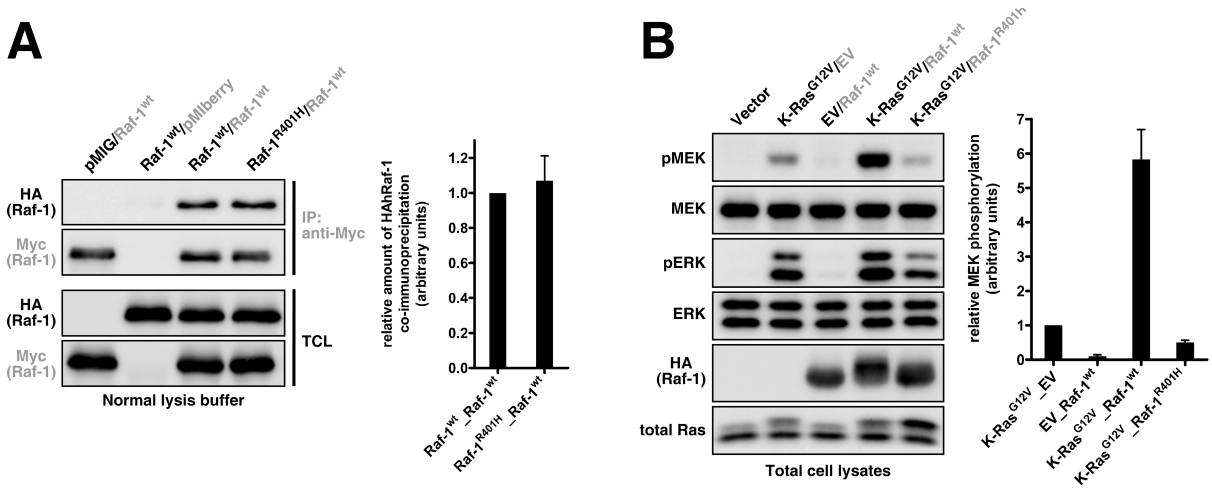

Figure 1 K-Ras^{G12V} induced Raf-1 activation is DIF dependent although the R401H mutation does not abrogate Raf-1 homo-dimer formation. **(A)** The indicated HA- and Myc-tagged Raf-1 constructs were co-expressed in Plat-E cells and purified using anti-Myc (9E10) antibodies. Immunecomplexes and total cell lysates were analysed by Western blotting (left). Bar graph showing the ratio of co-purified HA-Raf-1/precipitated Myc-Raf-1 (right). Data represent the mean ± S.E.M. from three independent transfections. Please note that the R401H mutant represents the Raf-1 equivalent of B-Raf^{R509H}. **(B)** The indicated HA-tagged Raf-1 constructs were co-expressed with K-Ras^{G12V} or empty vector (EV) in Plat-E cells and total cell lysates were analysed by Western blotting (left). Bar graph representing the mean MEK phosphorylation ± S.E.M. from three independent transfections (right).

4. This study shows that mutations in the DIF motif are efficient at preventing the formation of BRAF homodimers however they do not affect the formation of RAF heterodimers which are known to be important drivers of RAF paradoxical activation upon treatment with RAF inhibitors. This fact might undermine the use of DIF mutations as a strategy to prevent RAF paradoxical activation. Can the authors comment on this point?

The reviewer raises an important and timely question. We think that our manuscript provides the first dataset that addresses the possibility of targeting the human Raf DIF to potentially prevent paradoxical ERK activation in patients. In the original manuscript, we have shown that sorafenib-induced B-Raf hetero-dimers with A-Raf and KSR are abolished by the R509H mutation, while only the 3x mutation completely abolished B-Raf/Raf-1 dimers (Figure 7). We have also demonstrated, however, that paradoxical ERK activation by B-Raf$^{D594A/R509H}$ is completely abrogated although the former still dimerises efficiently with Raf-1 (Figure 6C and D). Thus, it appears that paradoxical ERK activation requires not only dimerisation, but in addition, transactivation, presumably via the DIF. These data show for the first time that dimerisation and transactivation can be separated from another.

In the revised manuscript, we address inhibitor-induced paradoxical ERK activation in more detail. Using our MEF complementation system in combination with the two structurally distinct inhibitors PLX4720 and L779450, both known to induce paradoxical ERK activation (Heidorn et al., 2010; McKay et al., 2011), we show that re-introduction of B-Raf^{wt} into B-Raf$^{-/-}$ MEFs further enhances the drug-induced paradoxical ERK activation, while the B-Raf^{R509H} and B-Raf^{3x} DIF mutants behave neutral or slightly dominant-negative. In the case of PLX4720, loss of paradoxical activation appears to be a combination of reduced B-Raf/Raf-1 hetero-dimerisation (less PLX4720-induced B-Raf^{R509H}/Raf-1 than B-Raf^{wt}/Raf-1 hetero-dimers) and loss of transactivation (compared to the amount B-Raf^{wt}/Raf-1 hetero-dimers in DMSO treated MEFs, there is still a similar amount of PLX4720-induced B-Raf^{R509H}/Raf-1 dimers available). These data strongly suggest that interfering with DIF function could be potentially exploited therapeutically, e.g. in conjunction with vemurafenib as it was suggested by Lavoie & Therrien (2011) and in our original manuscript.

Minor comments:

1. In Figure 1 the authors should present the quantification of MEK phosphorylation for all the mutants studied and not only just for a few selected ones.

We provide now quantification for all shown Western blot data in Figure 1 within the new Figure 1G. The B-Raf^{Q257R} mutant including quantification of the R509H effect is now presented in Supplementary Figure S2.

2. In Figure 1C and 1E the mutant R509H should be included as a control.
Although we appreciate the reviewer comment, we feel that the R509H mutant has been already included in multiple panels. We have analysed many B-Raf mutants in our present work and sometimes we had simply not enough lanes available. However, we provide now a new Supplementary Figure S1 (see below) that compares the R509H mutant with the EVKD and V600E mutants. We think that this new supplementary figure, together with our statistical analysis in Figure 1G allows for a thorough comparison of B-Raf^{R509H} with other mutants.

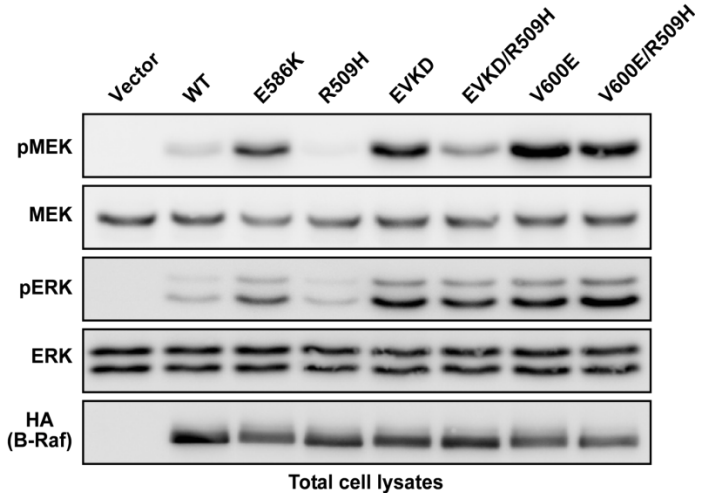

Figure 2 Unlike B-Raf^{wt} and B-Raf^{EVKD}, the MEK phosphorylation potential of B-Raf^{V600E} is hardly affected by the R509H DIF mutation. The indicated HA-tagged B-Raf constructs were expressed in Plat-E cells and analysed by Western blotting of total cell lysates with the indicated antibodies.

3. The mutants EVDK, EVDK/R509H, V600E, V600E/R509H are repeated in Figure 1C and 1E.
Although we agree with the reviewer that these data are repeated, we feel that these samples represent useful references to assess the signalling potential of the other g-o-f mutants and we would therefore prefer to leave these panels as they are.

4. Figure 6C should include an immunoblot for RAS to show that its expression is being efficiently induced by 4-hydroxy-tamoxifen.
We would like to thank the reviewer for this important suggestion. However, we would like to emphasise that the ERTMRas^{G12V} fusion protein is constitutively expressed in MEFs, but, in the absence of 4-hydroxy-tamoxifen (4-HT), remains shielded by heat shock protein complexes (Dajee *et al.,* 2002). Consequently, an immunoblot, which we now provide in the revised manuscript, shows expression of this fusion protein at all times. However, the administration of 4-HT can be indirectly visualized by the increase of the ERTMRas^{G12V} fusion protein in the samples derived from all 4-HT treated cells. This is a known phenomenon for estrogen receptor (ER) fusion proteins and is caused by the stabilisation of the ER ligand binding domain by 4-HT (Jin and Woodgett, 2005; Kamogawa et al., 1998; Woods et al., 1997).

5. In Figure 6C the authors present two immunoblots for BRAF by using one for the HA-tagged BRAF and another one for the endogenous protein (F7) but one of them should be removed as it does not add to the study.
We would like to thank the reviewer for this suggestion and simplified the figure by removing the anti-HA detection and left the F7 panel as it confirms the absence of endogenous protein in the knock-out MEFs.

6. In Figure 7B the levels of immunoprecipiated BRAF protein should be similar for all the analysed samples.

We thank the reviewer for this critical comment. As we have performed these experiments several times, we have now replaced this Figure by a very similar experimental set-up that also includes PLX4720, as suggested by reviewer #3. This and other findings confirm the data from the original figure 7B. However, the original figure 7B featured the difference between sorafenib-treated MEFs with and without oncogenic Ras signalling and therefore we transferred this figure into Supplementary Figure S5.

7. The first sentence of the Results section lacks reference.
We apologise for this oversight and have added (Rajakulendran *et al*., 2009) at the end of this sentence.

8. Some of the supplementary references are already included in the main text.
As we regard the main article and the supplementary dataset as two entities, which can be downloaded separately and are subject to distinct access requirements, we felt that it would be better if each document is fitted with a complete reference set referring to the data in each document. However, we are happy to modify this at the editor's discretion.

Referee #2

Raf proteins are part of the Ras/Erk pathway. Their Ras-dependent activation entails the dimerization of their kinase domain mediated by an evolutionarily conserved interface called the "side-to-side" interface.

In this paper, Roring et al. investigate the importance of the B-Raf dimerization interface to support the activity of various gain-of-function mutations that have been identified over the years. The central take-home message of this study is that the most frequent oncogenic mutation (V600E) found in human tumors is resilient to mutations impeding dimerization whereas WT B-Raf as well as other classes of gain-of-function mutants heavily depend on it. This information is potentially important as it could have an impact on the development on a new generation of B-Raf inhibitors to tackle B-RafV600E-dependent cancers. Unfortunately, this work suffers from a number of important shortcomings that significantly reduces its significance and scope.
We thank referee #2 for his/her criticism. We would like to emphasise at this point that the original and the revised manuscript offers more new insights into Raf signalling and regulation than the aforementioned "central take-home message" suggested by reviewer #2. This was also appreciated by reviewer #1 and #3. Our point-by-point response to the comments is provided below.

Major points:

1. Although the mechanism remains unknown, the apparent resilience of B-RafV600E to interface disruption has now been reported by another group (Poulikakos et al. 2011 Nature, Nov 23; Epub ahead of print).
We have also noticed the recent paper from the Solit/Rosen laboratory, which was published in *Nature* while our manuscript was under review (Poulikakos et al., 2011). This publication has identified a B-Raf^{V600E} splice mutant with enhanced dimerisation potential in cell lines and patients treated with the B-Raf inhibitor vemurafenib (PLX4032). This tumour-associated aberrant splice product confers resistance against this clinically highly relevant drug. The only overlap with our study is their observation that the MEK/ERK phosphorylation potential of B-Raf^{V600E} is not or, as our quantitative analyses (Figure 1G of the revised manuscript) rather show, hardly affected by the R509H DIF mutation. Transformation assays using DIF mutants as we provide them in Figure 5 are absent in the *Nature* paper. Furthermore, while the *Nature* paper shows that B-Raf^{V600E} can form homo-dimers, they are not studying other oncogenic mutants such as B-Raf^{G469A} and, importantly, do not include B-Raf^{wt} in their analysis. Consequently, our finding that B-Raf^{V600E} and B-Raf^{G469A} represent more potent homo-dimerisers than B-Raf^{wt} remains novel. We discuss our findings in the context of the *Nature* paper on p. 21/22 of the revised manuscript. Furthermore, our manuscript contains many additional and quantitative data on the function of the Raf dimer interface, such as its relevance for paradoxical ERK activation by kinase-dead and drug-bound B-Raf as well as for K-Ras^{G12V} induced Raf-1 signalling. These aspects are not featured in the *Nature* publication at all.

2. The claim that the highly oncogenic B-Raf V600E mutation does not require dimerization for activity is purely based on overexpression experiments (including the transformation assays), which may have confounding consequences.

First and foremost, we have not claimed that oncogenic B-Raf^{V600E} does not require dimerisation for activity, but wrote "*B-Raf^{V600E} [does] not require an intact DIF for efficient signalling*". Although our data and the recently published paper by Poulikakos et al. (2011) indirectly suggest that B-Raf^{V600E} can signal as a monomer, we have also written in the original and revised manuscript: "*Secondly, B-Raf^{V600E} could homo-dimerise independently of an intact DIF.*". Moreover, we have even discussed in the original manuscript how B-Raf^{V600E} could profit from its enhanced homo-dimerisation potential. Please see also our model in Figure 8D of the revised manuscript.
Firstly, although we agree that overexpression can sometimes cause artifacts, this argument cannot simply be applied here as we compare the functional characteristics of various B-Raf mutants at comparable expression levels. The fact that we, as well as reviewer #1 and #3, can discern and measure differences between the various B-Raf mutants in a quantitative and reproducible manner assures us that we are not reporting simple overexpression artifacts.
Secondly, as we have already pointed out above, it is very difficult not to make use of ectopically expressed, recombinant proteins in order to dissect molecular mechanisms. All recent publications in *Cell*, *Nature*, *PNAS* etc. have used exclusively ectopic overexpression experiments to investigate inhibitor-driven Raf dimerization, in many cases even using isolated Raf kinase domains. In contrast, our study uses full-length B-Raf and Raf-1 proteins. We also address the interaction of recombinant full-length B-Raf with endogenous Raf-1, A-Raf and KSR1 in our assays, which is also not that often seen in high impact publications concerned with Raf dimerisation.
Thirdly, we would like to stress that we have deployed two B-Raf deficient cell types, Raf-1/B-Raf double deficient DT40 cells and B-Raf deficient MEFs, in which we restored the expression of B-Raf or mutants thereof. The results from these complementation approaches support our Plat-E transfections experiments very well.
Fourthly, over-expression of B-Raf^{V600E} as a consequence of oncogene amplification has been reported for various tumor entities such as colon carcinoma (Corcoran et al., 2010; Little et al., 2011).
Lastly, using a combinatorial approach of allele-specific shRNA-mediated knockdown and a novel V600E-specific monoclonal antibody, we provided a data set showing for the first time that endogenously expressed B-Raf^{V600E} forms large signaling complexes in the human colon carcinoma cell line HT29 (Figure 4). Thus, our Plat-E cell system allowed us to discover a novel facet of oncogenic B-Raf^{V600E}, which is now confirmed for the endogenous oncoprotein in cells derived from a real human tumor.

3. Although the authors claim that the G469A mutation behaves as the V600E, which would be consistent with their model, the data shown in Fig. 1 are far from convincing as the R509H mutation seems to have a significant effect on it.
We thank reviewer #2 for this comment and we have carefully revisited all text passages involving the G469A mutant. Nevertheless, we have never claimed that the G469A mutation behaves exactly as V600E, which, by the way, also displays a statistically significant reduction in MEK phosphorylation capacity (p= 0.008, see Table S2 of original manuscript). However, there are clearly strong similarities between both oncoproteins. In that regard, it should be noted that others have also observed similarities between B-Raf^{V600E} and B-Raf^{G469A}, for example in terms of their independence from C-terminal 14-3-3 binding (Ritt et al., 2010).
Reviewer #2 is right that the R509H mutation has a significant effect on B-Raf^{G469A}. Compared with the other high activity mutants B-Raf^{V600E} and B-Raf^{insT}, it represents the most affected, although the R509H mutation reduces the MEK phosphorylation potential of B-Raf^{G469A} by only 39%. (See Supplementary Table S2 for mean values of all mutants presented in Figure 1). We do not think that these data contradict our model, as it is quite conceivable that the different mutations disrupting the hydrophobic interaction between P- and activation loop can also have additional effects that modulate the overall conformation of the kinase and its activity. For example, the V600E mutation is supposed not only to disrupt the aforementioned hydrophobic interaction, but also forms a novel salt-bridge between E600 and K507 within the Ca-helix, which in turn stabilises the active conformation (Wan et al., 2004). Such a salt-bridge cannot be formed by B-Raf^{G469A}.

4. Even if commonly used, immunoprecipitations and BN-PAGE assays remain artificial approaches to monitor protein-protein interactions. There is no guarantee that the results obtained by these

methods faithfully recapitulate the state of an interaction within a living cell. Given the higher ability of the V600E mutant to form dimers, it may well be that the apparent resilience of the V600E mutant to the disruption of the dimer interface is due to the fact that dimerization still proceeds to a low but significant level to support substantial MEK/ERK activation.

We are aware that IPs, as every experimental perturbation of a system, have limitations. However, Co-IP assays represent the current standard method in the field to monitor Raf dimerisation including the latest *Nature* paper mentioned by reviewer #2 (Baljuls et al., 2011; Garnett et al., 2005; Hatzivassiliou et al., 2010; Heidorn et al., 2010; Karreth et al., 2009; McKay et al., 2011; Poulikakos et al., 2011; Ritt et al., 2010; Rushworth et al., 2006). Furthermore, we would like to stress that our IP data have been quantified from multiple IP experiments and that some of the BN-PAGE experiments were performed on native complexes of endogenous B-Raf.

We would like to point out that BN-PAGE, as here protein complexes are not bound by antibodies prior to Western blotting, represents a method that preserves endogenous multi-protein complexes. Indeed, many insights from BN-PAGE analyses were later confirmed by other methods, e.g. electron microscopy or BiFC as shown for example for antigen receptor complexes on T and B cells ((Schamel et al., 2005; Schamel and Reth, 2000; Yang and Reth, 2010) or the mitochondrial respiratory chain (Schagger et al., 1994).

Again, we have always agreed with the second sentence of point 4 by referee #2 as we have also written in the original and revised manuscript: "*Still, we cannot completely rule out that transient dimer formation between B-Raf^{V600E} protomers occur in a DIF- and 14-3-3-independent manner leading to MEK phosphorylation.*" This is also reflected by our model in Figure 8D of the revised manuscript.

5. The entire study stands out as phenomenological and as such does not provide much novel information. While mechanistic speculation as to how a V600E could escape the need for dimer formation is provided, no attempt to directly address this is made.

We think that the precise molecular mechanism by which V600E escapes the need for dimer formation represents the scope of a manuscript in its own right. We would like to stress that Poulikakos *et al*., (2011) also do not provide such a mechanism and, probably due to the brevity of *Nature* letters, do not even provide a discussion of potential mechanisms as we do.

After we discovered that B-Raf^{V600E} could signal in the absence of an intact DIF, we decided to analyse the role of the DIF in more detail in wildtype B-Raf and a broad panel of its gain-of-function mutants. Although this approach might appear phenomenological at first glance, it allowed us at least to discuss as to why DIF mutations have such a little impact on B-Raf^{V600E}.

Furthermore, we feel that our study provides novel and interesting information as it was also appreciated by referees #1 and #3. For example, a recent commentary raised the question whether blocking DIF function could be used to treat Ras-driven cancers (Lavoie and Therrien, 2011). We showed in the original manuscript that B-Raf DIF mutants cannot be efficiently activated by oncogenic Ras and extend now these findings to Raf-1 as well. Furthermore, we have provided strong evidence in the original manuscript that an intact DIF is not required for the interaction between kinase-dead B-Raf and Raf-1, but for the activation of the latter. To the best of our knowledge, such data have not been published elsewhere.

6. Even though the authors conducted one experiment to show that the system is not saturated when using the V600E mutant (Fig. S1). They have to repeat those experiments with a more extended dose-response curve using lower plasmid quantities (they should decrease the levels of the V600E construct to a point where they start seeing a significant reduction in pMAPK levels). Multiple determinations and quantification (including error bars) need to be provided. In fact, it appears that those mutants that are the least affected by the interface mutations are the ones that have the strongest activity. For this reason it becomes very important to conduct titration experiments to ensure that the system is not saturated. It remains though that they would then need to show that those active mutants are truly monomeric in vivo. Otherwise, it would remain impossible to conclude anything.

We would like to thank the reviewer for this helpful suggestion. In the revised manuscript, we have again titrated B-Raf^{V600E} expression levels by reducing the amount of transfected plasmid DNA to rule out that our model system is saturated by the expression of ectopic B-Raf^{V600E}. Using a lower amount of plasmid DNA, we noticed a significant reduction of pERK levels by more than 70% compared to those observed transfecting the pre-established amounts of the pMIG/HAh*BRAF*^{V600E} construct (Supplementary Figure S2A and below) However, the differential in the MEK phosphorylation potential of B-Raf^{V600E}, B-Raf$^{V600E/R509H}$ and B-Raf$^{V600E/3x}$ was in fact very similar to

that described in the original manuscript (Figure 1H) and, importantly, not largely increased under drastically reduced HA-B-Raf expression levels. This indicates that the minor differential between B-Raf^{V600E} and B-Raf$^{V600E/R509H}$ does not represent an artefact caused by system saturation. Given these new results, we think that the concern of system saturation should only be applicable, if we were making a statement that these mutants are more active than B-Raf^{V600E}.
Lastly, we would like to emphasise again that we have never claimed that B-Raf$^{V600E/R509H}$ can function as a truly monomeric enzyme ("*Secondly, B-Raf^{V600E} could homo-dimerise independently of an intact DIF.*").

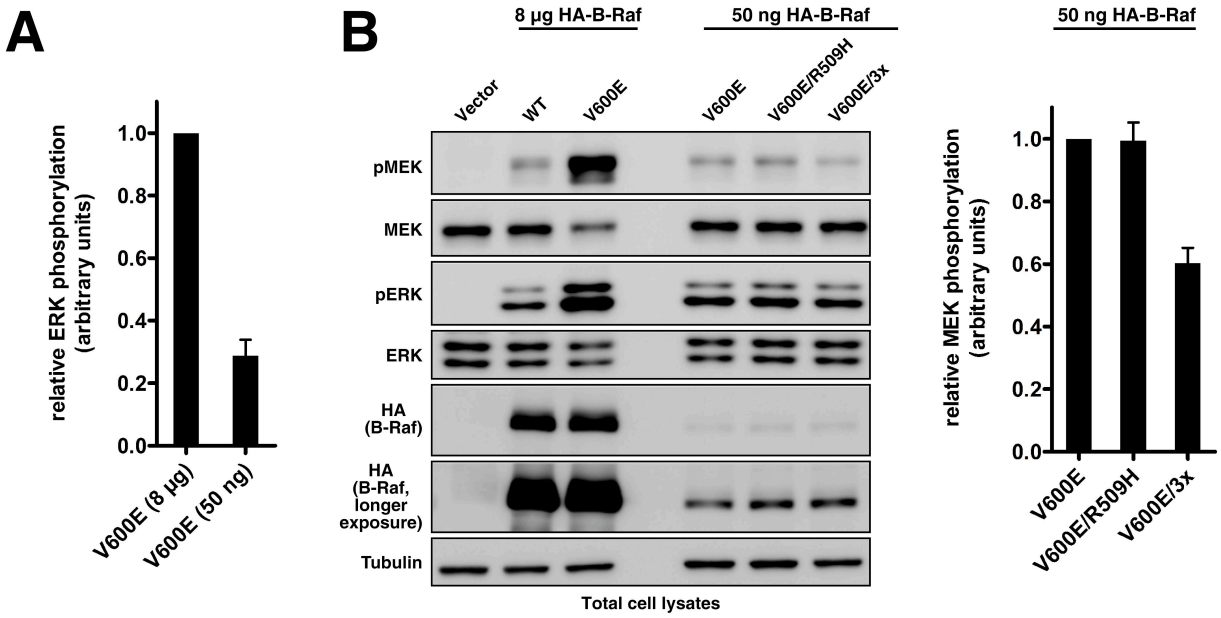

Figure 3 The minor impact of DIF mutations on the B-Raf^{V600E} MEK phosphorylation potential is not based on saturation of the experimental system. **(A)** Bar graph showing the relative level of ERK phosphorylation after transfecting 8 µg or 50 ng of the pMIG/HAh*BRAF*^{V600E} construct. Shown data represent the mean ± S.E.M. from five independent transfections (normalised on tubulin). **(B)** The indicated HA-tagged B-Raf constructs were expressed in Plat-E cells and analysed by Western blotting of total cell lysates with the indicated antibodies (left). A bar graph representing the mean MEK phosphorylation differential between B-Raf^{V600E}, B-Raf$^{V600E/R509H}$ and B-Raf$^{V600E/3x}$ ± S.E.M. from five independent transfections (normalised on HA-B-Raf) is shown on the right. Please note that each 50 ng HA-B-Raf DNA reaction was supplemented with 7.95 µg empty vector DNA to ensure equal amounts of total DNA per transfection.

Minor points;

7. At places, the authors simplify the literature. For example, on page 4; 2nd para.; line 7. They say that phosphorylation of the TVKS motif of the B-Raf activation loop leads to its restructure and cite Wan et al. 2004. There is actually no single study that demonstrated this. The Wan et al paper simply conjectured that this might happen, but they never showed it. The same statement is made on top of page 7. As a matter of fact, the phosphorylation of the indicated residues has been documented by a single group and never been reproduced (which make some researchers in this field uncomfortable with this information).
We would like to thank referee #2 for this important comment. We are well aware that, due to its disorder, the activation loop and consequently the TVKS-motif, have not been resolved in the X-ray structure analysis by Wan et al. (2004). Although, we think that there is strong support for the "restructuring model" from multiple angles and other groups have reported TVKS-motif phosphorylation in the meantime (Borysova et al., 2008; Chadee and Kyriakis, 2004), we agree that our original sentence was an oversimplification and have rephrased it accordingly:
"For example, wildtype B-Raf (B-Raf^{wt}) is stringently controlled by the Ras-dependent phosphorylation of its activation loop ($T^{599}VKS^{602}$-motif; Zhang and Guan, 2000). This supposedly restructures the catalytic center and induces kinase activity (Wan et al. 2004)."

8. The authors make multiple claims of being the first to demonstrate specific points:
p. 9; top line
p. 10; top line
p, 10; 2nd parag. Line 5
p. 15; 1st parag., line 7
p. 19; third line from the bottom
These are unnecessary and a bit annoying (an original paper provides by definition novel information).
In a rapidly developing field like this one, we felt that it would be important to stress what findings are novel in comparison to other recent papers on this topic (see also our comments to points 1 and 5 of reviewer 2). But we do agree that we have been a bit too repetitive and have rephrased some of these sentences.

Referee #3

In this paper the authors carefully dissect the role of B-Raf homo- and heterodimerization to the activation of the ERK pathway and oncogenic transformation. The paper is well organized, the experiments are generally convincing, and the conclusions are interesting for the field. The results demonstrate that the role of Raf dimerization is much more complicated than previously thought. I just have a few comments that may serve to improve the paper.
We would like to thank reviewer #3 for the valuable comments.

Major points
Fig. 1A. A recent publication by Baljuls et al. (J Biol Chem. 2011 May 6;286(18):16491-503) shows that the three amino acids immediately N-terminal of the DIF are also involved in Raf dimerization. The authors may consider extending their definition of the DIF motif to include these amino acids.
Although we have considered the publication by Baljuls *et al*., (2011) at a different position in the original manuscript, we would like to thank the reviewer for this suggestion. Consequently, we have slightly extended the introductory paragraph on p. 4, which accompanies Figure 1B.

Fig. 4. In the previous publications about the paradoxical activation of the ERK pathway by Raf inhibitors the effects of PLX4032 tended to behave different than those of sorafenib. Thus, a comparison of PLC4032 and sorafenib in regard to the DIF mutants would be very interesting.
We would like to thank reviewer #3 for this interesting and important suggestion. In addition to sorafenib, we have now tested the behavior of the DIF mutants in our MEF complementation system treated with PLX4720 (the tool compound for PLX4032/vemurafinib) and L779450. As shown previously by other groups (Heidorn et al., 2010; McKay et al., 2011; Poulikakos et al., 2010), both inhibitors cause paradoxical MEK/ERK phosphorylation in B-Raf deficient MEFs reconstituted with B-Raf^{wt}. Most importantly, however, we show that this effect is not observed if the knock-out MEFs are complemented with the DIF mutants B-Raf^{R509H} and B-Raf^{3x} (Figure 7 of the revised manuscript and below). Without going in too much detail here, we also think that our data might reconcile opposing views on the mechanisms by which Raf inhibitors cause paradoxical MEK/ERK activation (see (Cox and Der, 2010; Wimmer and Baccarini, 2010) for discussion of the primary literature). These new data are now incorporated into Figure 7 and are being described and discussed in detail in the revised manuscript.

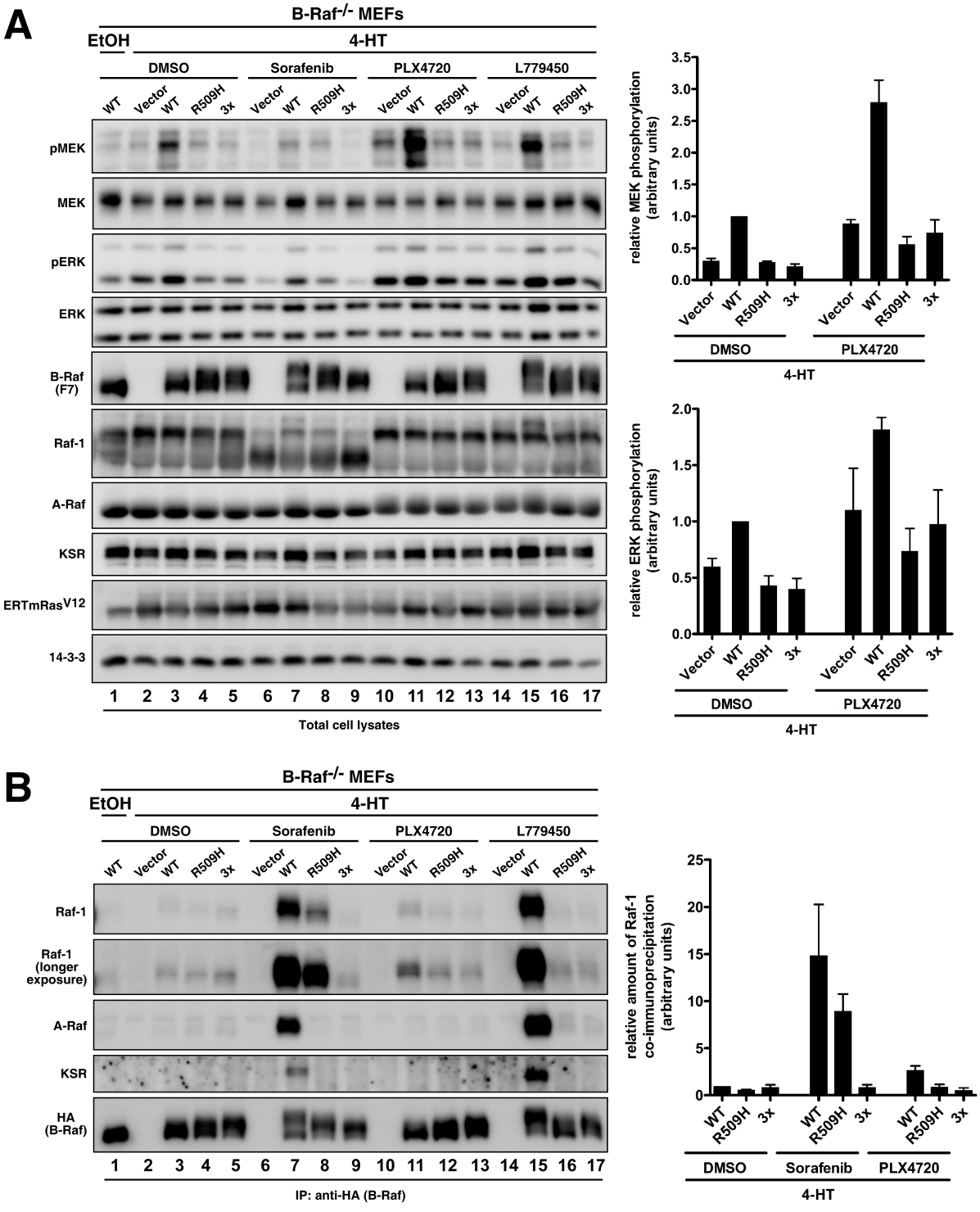

Figure 4 Raf inhibitor-induced paradoxical ERK activation is DIF-dependent as is concurrent protein complex formation of HA-B-Raf with endogenous Raf-1, A-Raf and KSR. The indicated HA-B-Raf constructs were expressed in the MEFs described in (6A) and purified with anti-HA (3F10) antibodies from normal lysis buffer (NLB) lysates. Prior to lysis, the MEFs were treated with 4-HT or vehicle (EtOH) followed by sorafenib (10 μM), PLX4720 (1 μM), L779450 (1 μM) or vehicle (DMSO) for 4 h. Total cell lysates **(A)** and immunecomplexes **(B)** were analysed by Western blotting with the indicated antibodies. Shown bar graphs represent the mean ± S.E.M. from three independent transductions.

Fig. 7B. These results suggest that the binding sites for homo- and heterodimerization are different. This could allow the formation of ternary or even higher order complexes including homo- and heterodimers. Is that the case?

Reviewer #3 raises an important question here, which we have also discussed extensively among ourselves, in particular as B-Raf^{V600E}, which we found to possess enhanced homo-dimerisation potential, is more likely to exist in larger protein complexes in our BN-PAGE analyses (Figure 4). Based on published data on the stoichiometry of abundant B-Raf signalosome components (Catalanotti et al., 2009; Vaughan et al., 2006) and our own unpublished SILAC data confirming Hsp90/Cdc37, MEK and 14-3-3 as more or less constitutive interaction partners of B-Raf complexes, one could speculate that these larger complexes could comprise a B-Raf homodimer (2 x 95 kDa) with each protomer bound to a Hsp90/p50^{Cdc37} complex (2x 90 kDa + 50 kDa) as well as a MEK (2 x 50 kDa) and one or two 14-3-3 dimers (2 – 4 x 30 kDa). In contrast, the bulk of B-Raf^{wt} is found in smaller complexes, while only a small fraction occurs in larger complexes. This is also in agreement with the reported size of 250 kDa for B-Raf homodimers (Rushworth et al., 2006). Thus, if our rough calculations were right, one would rather estimate that one homo- or hetero-dimer is at the centre of the core signalling complex consisting of HSP90/Cdc37, MEK and 14-3-3. Nevertheless, the formation of ternary or even higher complexes, for example in a transient manner during growth factor stimulation, cannot be excluded and remains an attractive idea. Although we think that addressing this point is beyond the scope of the present manuscript and represents an area for future studies, we hope that our present data showing the formation of large B-Raf complexes and the different rules for homo- and hetero-dimerisation will stimulate research in this direction.

It would be interesting to compare the effects of some of the DIF mutants in cell lines, such as melanoma, where the Raf inhibitors have been previously studied.

We would like to thank reviewer #3 for this interesting suggestion. To this end, we resorted to SBcl2 cells, a human melanoma line endogenously expressing oncogenic N-Ras^{Q61K} (Satyamoorthy *et al.*, 2003), which has previously been shown to exhibit paradoxical ERK activation upon PLX4720 treatment (Kaplan et al., 2011). In order to facilitate transductions with murine retroviruses carrying HA-tagged wildtype B-Raf and mutants thereof, these cells were transiently transfected with a plasmid encoding an ecotropic receptor. In agreement with the results obtained in our MEF system (see Figure 4 above) and despite the fact that Sbcl2 cells express endogenous wildtype B-Raf, ectopic expression of B-Raf^{R509H} reduces paradoxical MEK/ERK phosphorylation resulting from PLX4720 treatment below that of the vector control (Supplementary Figure S7 and below). We are aware that the expression level of B-Raf^{R509H} is lower than that of B-Raf^{wt} (Supplementary Figure S7 and below). Importantly, however, slightly higher B-Raf^{R509H} expression levels compared to ectopic B-Raf^{wt} appear to have a very similar effect on PLX4720-induced paradoxical MEK/ERK activation, at least in our MEF system (see Figure 4 above). This seemingly dominant-negative effect of B-Raf^{R509H} is also in line with data in the original manuscript showing that B-Raf^{R509H} suppresses MEK/ERK activation in EGF-treated (Figur 2A) and active ERtmHRas^{V12} expressing MEFs.

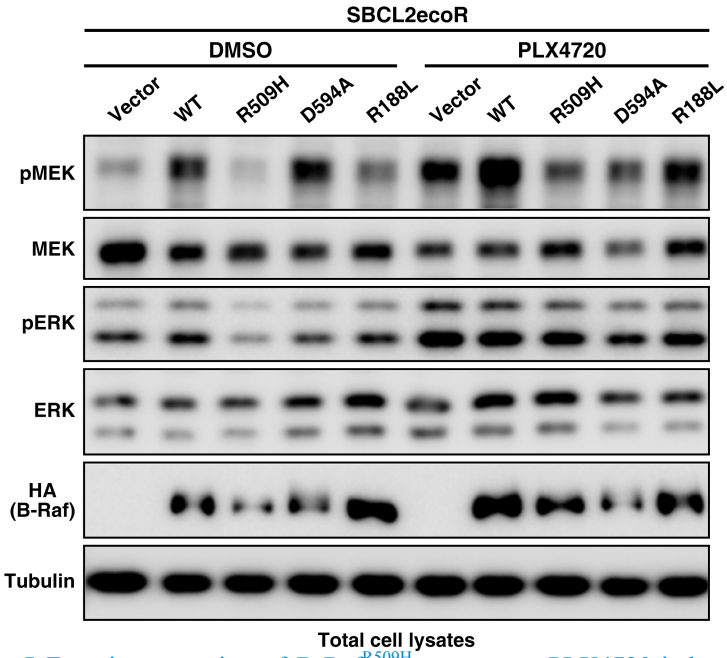

Figure 5 Ectopic expression of B-Raf^{R509H} suppresses PLX4720-induced paradoxical MEK/ERK activation in the human *NRAS*^{Q61K}-mutant melanoma cell line SBcl2, which was previously shown to exhibit paradoxical ERK activation upon PLX4720 treatment (Kaplan et al., 2011). The indicated HA-tagged B-Raf constructs were transduced into SBCL2 cells transiently expressing an ecotropic receptor (SBCL2ecoR) facilitating infections with murine retroviruses. Please see supplementary methods for details. Following treatment with DMSO (vehicle) or 1 µM PLX4720 for 4 h, total cell lysates were analysed by Western blotting with the indicated antibodies. Please note that, despite the lower expression levels of B-Raf^{R509H} compared to ectopic B-Raf^{wt}, the former already possesses sufficient dominant-negative activity to decrease the resulting levels of MEK/ERK phosphorylation below those of the vector control.

Minor points

p.3. "The RBD mediates the interaction with Ras-GTP through a conserved arginine residue (R188 in B-Raf)." This sentence should be changed as in the current form it suggests that R188 is the sole mediator of the RBD-Ras interaction, which is not correct.

We would like to thank reviewer #3 for this important comment and have changed the sentence accordingly: “The RBD mediates the interaction with Ras-GTP. A conserved arginine residue (R188 in B-Raf) in the RBD is required for the recruitment and activation of Raf at the plasma membrane as well as for dimerisation with Raf-1 (Heidorn et al., 2010; Marais et al., 1997).”

p.3. "Displacement of 14-3-3 from the CR2 and subsequent dephosphorylation of S365 (or its equivalent) is a key step in Raf activation (Rodriguez-Viciana et al., 2006a)." This already has been shown previously by Abraham et al., J Biol Chem. 2000 Jul 21;275(29):22300-4; Jaumot & Hancock, Oncogene. 2001 Jul 5;20(30):3949-58; Dhillon et al., EMBO J. 2002 Jan 15;21(1-2):64-71.

We would like to thank reviewer #3 for this suggestion. We are very familiar with these important studies and had them even incorporated in an earlier draft, but removed them to keep the manuscript succinct. For this reason, we focussed on the Rodrigues-Viciana paper as this was the first paper showing the regulation of B-Raf by dephosphorylation. However, as EMBO Journal does no longer limit the number of references and as our sentence eludes to Raf in general, we have cited these papers now as well.

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2nd Editorial Decision 21 March 2012

Thank you for the submission of your revised manuscript to The EMBO Journal. It has been sent to one of the original reviewers, who now considers that his/her concerns have been properly addressed and your manuscript is ready for publication.

Thank you very much again for your patience and congratulations on a successful publication.

Yours sincerely,

Editor
The EMBO Journal

